Tubulin Adenosine Diphosphate Ribosylation Is Catalyzed by Cholera Toxin†

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ABSTRACT: Cholera toxin catalyzed the transfer of radioactive label from [*adenine*-2,8-$^3H_2$]NAD^+ or [^{32}P]NAD^+ to rat C6 glioma cell membrane and cytosolic proteins. Labeled proteins were resolved by polyacrylamide–$NaDodSO_4$ gel or two-dimensional gel electrophoresis and stained with Coomassie blue, and the gels were subjected to fluorography or autoradiography. Autoradiograms of gels revealed labeled M_r 42 000 and 46 000–48 000 membrane proteins that are putative subunits of the regulatory component (G/F) of the C6 cell hormone-sensitive adenylate cyclase. Cholera toxin also catalyzed the labeling of several cytosolic proteins including a M_r 54 000 protein that was observed in autoradiograms of two-dimensional gels to migrate as an acidic satellite relative to Coomassie-stained C6 cell tubulin. Tubulin modified by ADP-ribosylation would undergo an acid shift relative to the stained unmodified tubulin in two-dimensional gels. The data led us to postulate that tubulin undergoes cholera toxin catalyzed ADP-ribosylation. Bovine brain tubulin prepared by three cycles of warm/cold polymerization/depolymerization was incubated with [^{32}P]NAD^+, GTP, and cholera toxin and then subjected to two-dimensional gel electrophoresis. Autoradiograms of the gels revealed the presence of [^{32}P]ADP-ribosylated proteins that migrated as acidic satellites relative to the Coomassie-stained brain α and β tubulin. Peptide maps of bovine brain tubulin and the associated [^{32}P]ADP-ribosylated proteins showed a correspondence between the autoradiographic images and the stained peptide fragments. The data demonstrate that cholera toxin catalyzes the ADP-ribosylation of tubulin.

Cholera toxin catalyzes the covalent modification of proteins by an ADP-ribosyl moiety derived from NAD^+ (Moss & Vaughan, 1979). Most notably, cholera toxin catalyzes the ADP-ribosylation[1] of a guanyl nucleotide binding regulatory protein termed G/F (Northrup et al., 1980; Ross & Gilman, 1980) or N (Kaslow et al., 1981a) that is required for the coupling of hormone receptors to adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. ADP-ribosylation of the G/F protein leads to the inhibition of an associated GTPase and the concomitant alteration of adenylate cyclase activity (Cassel & Selinger, 1977). In addition to peptide subunits of the G/F protein, there are unidentified proteins that also undergo cholera toxin catalyzed ADP-ribosylation (Gill, 1979; Gill & Meren, 1978). ADP-ribosylation of these proteins is not known to influence adenylate cyclase activity. In the present report, we identify tubulin as a target for cholera toxin catalyzed ADP-ribosylation. A preliminary report of this work has appeared (Hawkins & Browning, 1981).

Materials and Methods

Materials. [*adenylate*-^{32}P]NAD^+ and [*adenine*-2,8-$^3H_2$]NAD^+ (10 000–50 000 and 3300 Ci/mol, respectively) were obtained from New England Nuclear. Tissue culture supplies and reagents for gel electrophoresis were obtained as described previously (Groppi & Browning, 1980). *Staphylococcus aureus* strain V8 protease was obtained from Miles Laboratories. Cholera toxin was obtained from Sigma.

Cell Culture and Subcellular Fractionation. Rat C6 glioma cells were grown in roller bottles in Ham's F-10 medium plus 10% fetal calf serum as described previously (Browning et al., 1974, 1976). C6 membranes and cytosol were prepared essentially by the method of Jett et al. (1977) except that the cells were loaded with glycerol in Ham's F-10 medium and allowed to stand 40 min at 0 °C before lysis. The cells were lysed and homogenized with 10 strokes of a B pestle in a Dounce homogenizer at 0 °C in 10 mM Tris (pH 7.4), 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (TMC lysis buffer) in a volume equal to 10% of the culture incubation volume. The lysed cell suspension was centrifuged at 700*g* for 5 min. The supernatant was saved, the crude nuclear pellet was washed twice in TMC lysis buffer, and the supernatants were saved. The pooled supernatants were then centrifuged at 95000*g* for 90 min onto a 38% sucrose cushion containing 10 mM Tris (pH 7.4). The supernatant above the membrane band was saved and designated "cytosol". The crude membrane fraction was purified by dilution to 4% sucrose with 10 mM Tris (pH 7.4) followed by centrifugation at 26000*g* for 95 min onto 38% sucrose. The band at the interface was saved and designated "washed membranes". The fraction earlier designated as cytosol was concentrated by ultrafiltration using an Amicon UM-10 filter.

Membrane Marker Enzyme Assay. The washed membranes were assayed for thymidine 5′-phosphodiesterase (EC 3.1.3.35) as a plasma membrane marker as described by Jett et al. (1977) using *p*-nitrophenylthymidine 5′-phosphate (pNPdT5P) as the substrate. Washed membranes hydrolyzed 0.4 μmol of pNPdT5P min^{-1} (mg of protein)$^{-1}$ at 37 °C in contrast to cellular homogenates that hydrolyzed 0.08 μmol of pNPdT5P min^{-1} (mg of protein)$^{-1}$ at 37 °C.

Brain Tubulin. Bovine brain tubulin prepared by three cycles of warm/cold polymerization/depolymerization by the method of Shelanski et al. (1973). The purity of the microtubule preparation was demonstrated when 15 μg of the microtubule protein was resolved by two-dimensional gel electrophoresis. Stained α and β tubulin can be seen in Figure 3A.

Incubation Conditions for Cholera Toxin Catalyzed ADP-ribosylation. Rat C6 glioma cell membranes and/or

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[1] Abbreviations: ADP-ribosylation, the covalent modification of a protein by an adenosine diphosphate ribosyl moiety; BSA, bovine serum albumin; cAMP, adenosine cyclic 3′,5′-phosphate; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; G/F, guanine nucleotide binding regulatory component of adenylate cyclase; LDH, lactic dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; pNP5dTP, *p*-nitrophenylthymidine 5′-phosphate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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cytosol or bovine brain tubulin (2 mg/mL) were incubated in 1.5-mL Eppendorf tubes at 35 °C for 30–60 min (as indicated) in a 150-μL final volume. Incubation media contained 10 mM thymidine, 1.4 mM $MgCl_2$, 4 mM dithiothreitol (DTT), 1.4 mM Na_2-ATP, 0.66 mM EDTA, 69 mM KH_2PO_4 (pH 7.4), and 1–50 μM [*adenylate*-^{32}P]NAD^+ or [*adenine*-2,8-$^3H_2$]-NAD^+ as indicated. GTP (0.9 mM) and cholera toxin (20–60 μg/mL) were added as indicated. Incubations were terminated in one of two ways: In initial experiments 17 μL of 100% trichloroacetic acid at 0 °C was added to make the samples 10% in Cl_3AcOH. The Cl_3AcOH-treated samples were allowed to stand on ice for 1 h before centrifugation. The Cl_3AcOH-insoluble material was washed twice with 1 mL of diethyl ether to extract Cl_3AcOH. In later experiments, incubations were terminated by the addition of 2.5 volumes of ethanol and the samples allowed to stand overnight at −20 °C before centrifugation. All centrifugations were for 2 min at 8000*g* with an Eppendorf microcentrifuge. The ethanol- and Cl_3AcOH-insoluble protein samples were solubilized for discontinuous polyacrylamide–$NaDodSO_4$ gel or two-dimensional gel electrophoresis as described below.

Gel Electrophoresis and Autoradiography. Two-dimensional gel electrophoresis was performed by the method of O'Farrell (1975) as previously described (Groppi & Browning, 1980) except that the sample pellets were not treated with S_1 nuclease but were sonicated (Sanders & Rutter, 1974) to ensure dispersal. Discontinuous polyacrylamide–$NaDodSO_4$ gel electrophoresis was performed according to Laemmli (1975) by using a linear–exponential polyacrylamide gradient as previously described (Groppi & Browning, 1980). Gels were stained with 0.2% Coomassie blue, destained, dried, and subjected to autoradiography as previously described (Groppi & Browning, 1980). Fluorography (Bonner & Laskey, 1974) was performed on gels containing ^{3}H-labeled proteins. The gels were dehydrated for 40 min in Me_2SO and then incubated for 40 min in Me_2SO containing 16% (w/v) of PPO (2,5-diphenyloxazole). The gels were then washed in water for 40 min to precipitate PPO and remove Me_2SO. The dried gels were then photographed and subjected to fluorography at −70 °C.

Peptide Mapping of Brain Tubulin. Peptide mapping of bovine brain tubulin was performed according to the method of Cleveland et al. (1977). After incubation of bovine brain tubulin with [^{32}P]NAD^+, GTP, and cholera toxin, samples were subjected to $NaDodSO_4$ gel electrophoresis (12% gel; 1.2 mm thick). The gels were stained with Coomassie blue for 30 min and destained for 1 h, and the stained tubulin band and the associated ^{32}P-labeled products were cut from the gel. The gel sample containing 20 μg of bovine brain tubulin was equilibrated with 10 mL of sample buffer containing 1 mM EDTA–20% glycerol–0.1% $NaDodSO_4$–125 mM Tris (pH 6.8) for 40 min with one change of buffer after 20 min. The gel sample was then macerated and loaded onto a second gel (8–19% linear–exponential gradient gel) with a 5% stacking gel (4 cm). The gel fragments suspended in the sample buffer were overlayed with 0.04 μg of *S. aureus* strain V8 protease in 20 μL of sample buffer modified to contain only 10% glycerol. The gel was electrophoresed at 10 mA until the bromphenol blue dye marker reached the bottom of the separation gel. The gel was stained, destained, dried, and subjected to autoradiography.

Results

Cholera toxin catalyzed the transfer of ^{32}P from [^{32}P]NAD^+ to M_r 42 000 and 47 000 proteins of C6 cell membranes (Figure 1, lane G vs. lane B). This labeling was stimulated by the addition of GTP (Figure 1, lane H vs. lane G). These products are most likely subunits of the regulatory guanyl nucleotide binding protein (G/F) of the hormone-sensitive adenylate cyclase of rat C6 glioma cell membranes. These labeled M_r 42 000 and 46 000–48 000 C6 cell proteins were observed in autoradiograms of two-dimensional gels of samples containing mixtures of membrane and cytosol or membrane alone (data not shown) but were not observed in samples containing cytosol alone (Figure 2). In two-dimensional gels, these cholera toxin dependent M_r 42 000 and 47 000 membrane proteins were observed to migrate similarly to the ^{32}P-ADP-ribosylated G/F peptide subunits previously characterized for other sources of G/F (Schleifer et al., 1980; Kaslow et al., 1981a). The M_r 47 000 product migrates as a series of charge variants with the most acidic species resolving in the same region as actin in the charge dimension. The M_r 42 000 product migrates to a more basic position than the M_r 47 000 protein.

C6 cell cytosol was incubated with cholera toxin and [^{32}P]NAD^+ under the same incubation conditions that resulted in labeling of C6 membrane G/F subunits. Several [^{32}P]-ADP-ribosylated proteins were detected in autoradiograms of $NaDodSO_4$ gels (Figure 1, lanes D and E) including M_r 53 000–55 000 cytosolic proteins (indicated by "b"). Similar labeling of M_r 53 000–55 000 proteins was not observed in samples containing membrane alone (Figure 1, lanes G and H) but was observed in mixtures of membrane and cytosol (Figure 1, lane J). The addition of GTP did not appreciably stimulate the labeling of M_r 53 000–55 000 substrates in assays containing C6 cytosol alone (Figure 1, lanes D and E).

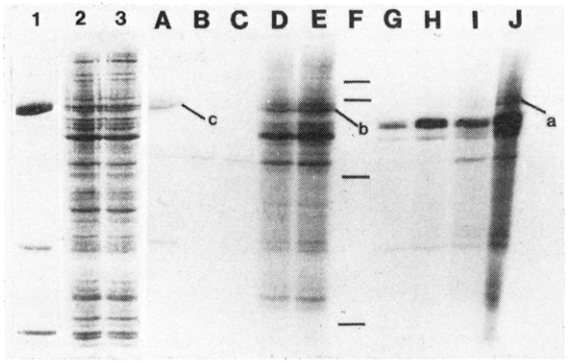

FIGURE 1: Autoradiogram of polyacrylamide–$NaDodSO_4$ gel of [^{32}P]ADP-ribosylated C6 cell proteins. C6 cell membrane (15 μg), C6 cytosol (64 μg), or mixtures of C6 cell plasma membranes (15 μg) and C6 cytosol (8 μg) were incubated with 5 μM [^{32}P]NAD^+ (2000 Ci/mol) in incubation buffer (see Materials and Methods), ± GTP, and ± cholera toxin at 35 °C for 45 min. Incubations were terminated by the addition of Cl_3AcOH as described under Materials and Methods. Protein samples were solubilized in 85 μL of sample buffer, and 20 μL of each sample was loaded onto a polyacrylamide–$NaDodSO_4$ gel (8–16% linear–exponential gel gradient). A bovine brain tubulin sample (8 μg) also incubated with cholera toxin, GTP, and [^{32}P]NAD^+ was loaded onto the gel in lane A, indicated by "c". Lane F contains molecular weight markers that are, from top to bottom, BSA at 68K, pyruvate kinase at 57K, LDH at 36K, and cytochrome *c* at 11.5K. Lane 1 is the Coomassie-stained brain tubulin corresponding to lane A of the autoradiogram. The lower molecular weight Coomassie-stained bands in lane 1 are the A_1, B, and A_2 subunits of cholera toxin known to be M_r 21 000, 11 600, and 7000, respectively (Holmgren, 1981). Coomassie-stained bands in lanes 2 and 3 are the Coomassie-stained C6 cytosol corresponding to lanes D and E in the autoradiogram. Labeled protein samples were loaded onto the remaining gel lanes as follows: lane B, C6 cell membrane/cytosol mixture; lane C, C6 cell membrane/cytosol mixture/+GTP; lane D, C6 cell cytosol/+toxin; lane E, C6 cell cytosol/+toxin and GTP; lane G, C6 cell membrane/+toxin; and lane H, C6 cell membrane/+toxin and GTP; lane I, C6 cell membrane/cytosol mixture/+toxin; and lane J, C6 cell membrane/cytosol mixture/+toxin and GTP.

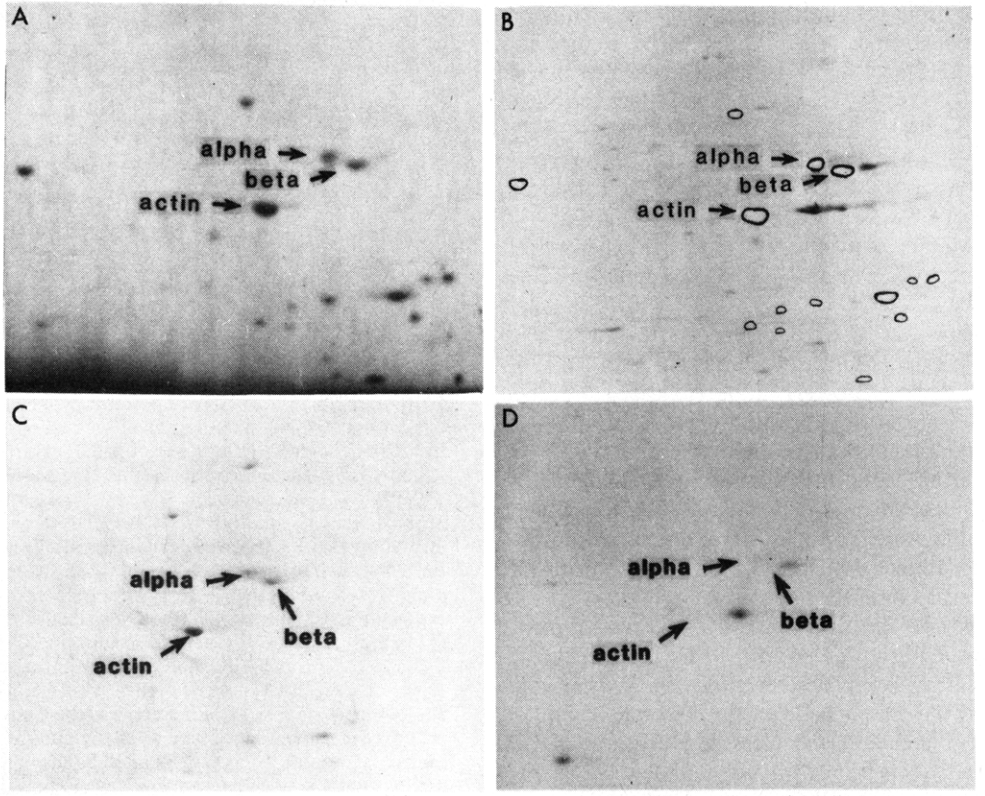

FIGURE 2: Autoradiograms of two-dimensional gels of C6 cytosol incubated with labeled NAD^+ and cholera toxin. (A) Coomassie-stained gel. C6 cytosol was incubated with [*adenine*-2,8-$^3H_2$]NAD^+ (20 μM), GTP, and cholera toxin (60 μg/mL) at 35 °C for 1 h and then ethanol-precipitated and subjected to two-dimensional gel electrophoresis as described under Materials and Methods. The gel was stained with Coomassie blue, then impregnated with PPO, dried, and photographed. (B) Autoradiogram. The gel in (A) was subjected to fluorography for 70 days at −70 °C, and the resulting autoradiogram is pictured in (B). Outlines have been drawn for reference to indicate the positions of stained C6 cell α and β tubulin and actin. A toxin-dependent labeled product can be seen to migrate as an acidic satellite to the right of C6 β tubulin. A much more faintly labeled satellite was observed to the right of α tubulin. Outlines have also been drawn to indicate the position of other abundant stained proteins that did not appear to undergo ADP-ribosylation. (C) Coomassie-stained two-dimensional gel of C6 cytosol incubated with [^{32}P]NAD^+, GTP, and cholera toxin. (D) 24-h autoradiogram of the gel in (C).

However, GTP did stimulate the labeling of M_r 53 000–55 000 substrates in assay mixtures containing both C6 membrane and cytosol (Figure 1, lanes I and J; indicated by "a"). The concentration of the cytosolic fraction present in assays containing both membrane and cytosol was 8-fold more dilute than that used in assays containing cytosol alone (Figure 1, lanes D and E). C6 tubulin was enriched in the cytosolic fraction as a likely consequence of cold-induced microtubule depolymerization during subcellular fractionation (Wiche & Cole, 1976; Groppi & Browning, 1980). Some other abundant stained bands corresponded to labeled bands in the one-dimensional gel (Figure 1, lanes 2 and 3 vs. lanes D and E); others did not. Examination of two-dimensional gels (Figure 2) for a similar relationship between stained proteins and ADP-ribosylated proteins did not bear out this association. Several proteins appeared to be ADP-ribosylated that were not sufficiently abundant to be detected by Coomassie staining (Figure 2). Consequently, toxin-catalyzed ADP-ribosylation was not strictly a function of the relative abundance of potential protein substrates, and in this sense some degree of substrate specificity was exhibited.

C6 cytosol was incubated with either [*adenine*-2,8-$^3H_2$]-NAD^+ or [^{32}P]NAD^+, GTP, and cholera toxin, and the resulting two-dimensional gels were subjected to ^{3}H fluorography or ^{32}P autoradiography (Figure 2). A labeled cytosolic protein was observed in autoradiograms to migrate as an acidic satellite relative to the Coomassie-stained C6 cell tubulin (Figure 2). This labeled product was also observed as an acidic satellite to tubulin of samples of C6 membrane/cytosol mixtures (data not shown) incubated similarly to samples in which ADP-ribosylation was stimulated by added GTP (Figure 1, lane J). Because ADP-ribosylation confers an increased negative charge on proteins, ADP-ribosylated proteins migrate to more acidic positions relative to unmodified proteins in electrofocusing gels. Our observations led us to postulate that C6 tubulin may be a substrate for cholera toxin catalyzed ADP-ribosylation. Therefore, we evaluated authentic brain tubulin as a possible cholera toxin catalyzed ADP-ribosylation product.

Bovine brain tubulin was incubated with cholera toxin, GTP, and [^{32}P]NAD^+ and subjected to $NaDodSO_4$ gel electrophoresis and autoradiography. [^{32}P]ADP-ribosylation was detected at M_r 53 000–55 000 (Figure 1, lane A, indicated by "c") coincident with the Coomassie-stained tubulin. Similarly incubated samples of brain tubulin were analyzed by two-dimensional gel electrophoresis. The presence of ^{32}P was observed in autoradiograms of the gels to migrate as acidic satellites relative to the Coomassie-stained brain α and β tubulins (parts A and B of Figure 3). When [*adenine*-2,8-$^3H_2$]NAD^+ was used instead of [^{32}P]NAD^+ and labeled proteins were visualized by fluorography, toxin-dependent ^{3}H-labeled products were again observed to migrate as acidic satellites relative to the Coomassie-stained brain tubulin (data not shown). The cholera toxin catalyzed transfer of label from either [*adenine*-2,8-$^3H_2$]NAD^+-labeled NAD^+ or [α-^{32}P]-NAD^+ to these substrates was consistent with the transfer of an ADP-ribosyl moiety (Moss & Vaughan, 1979). Furthermore, the modification of a small fraction of tubulin by ADP-ribosylation would produce the observed migration of the ^{3}H- or ^{32}P-labeled protein as an acidic satellite relative to unmodified Coomassie-stained tubulin. Approximately 0.1–1%

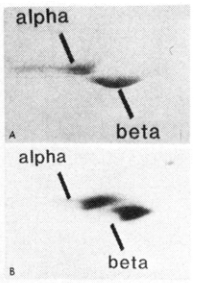

FIGURE 3: Two-dimensional gel of brain tubulin incubated with [^{32}P]NAD$^+$, GTP and cholera toxin. (A) Coomassie-stained gel. Bovine brain tubulin (2 mg/mL) was incubated with 5 μM [^{32}P]NAD$^+$ (20000 Ci/mol) and cholera toxin (20 μg/mL) and GTP for 30 min at 35 °C and ethanol-precipitated for two-dimensional gel electrophoresis (see Materials and Methods). Fifteen micrograms of tubulin was loaded onto the gel. α and β tubulins are indicated as such (α 1 above α 2; β 1 to the left of the more acidic β 2 tubulin). (B) Autoradiogram of gel in (A). The autoradiogram in (B) reveals that a small fraction of both α and β brain tubulin may have undergone toxin-dependent [^{32}P]ADP-ribosylation. The [^{32}P]ADP-ribosylated proteins were observed to migrate as acidic satellites to both the Coomassie-stained α and β brain tubulins. The autoradiogram was exposed beyond the linear range of the film.

of the tubulin was found to be ADP-ribosylated when 10^{-5} M brain tubulin was incubated with cholera toxin and 20 and 200 μM [^{32}P]NAD$^+$ and the labeled tubulin cut from stained 10% polyacrylamide–NaDodSO$_4$ gels and radioactivity was measured by liquid scintillation counting.

Finally, peptide mapping was performed to show that the toxin-dependent [^{32}P]ADP-ribosylated products were tubulin per se and not trace contaminants. Brain tubulin was incubated with cholera toxin, GTP, and [^{32}P]NAD$^+$ and purified by discontinuous polyacrylamide–NaDodSO$_4$ gel electrophoresis. The Coomassie-stained tubulin and the associated ^{32}P-labeled products were then cut from the gel and subjected to peptide mapping by the method of Cleveland et al. (1977) using *S. aureus* strain V8 protease. Correspondence was seen between the autoradiographic images and the Coomassie-stained peptide fragments of brain tubulin (Figure 4). The fact that the toxin-dependent [^{32}P]ADP-ribosylated peptide fragments and the Coomassie-stained peptide fragments of tubulin were observed to migrate together constituted strong evidence that tubulin is indeed a substrate for cholera toxin catalyzed ADP-ribosylation.

Discussion

Cholera toxin catalyzed the ADP-ribosylation of M_r 42000 and 46000–48000 membrane proteins that are likely polypeptide subunits of the G/F protein of the hormone-sensitive adenylate cyclase of rat C6 glioma cells. It was concluded that these polypeptides are subunits of the C6 cell G/F protein because of the following similarities to G/F from other sources (Watkins et al., 1981; Enomoto & Gill, 1980; Moss & Vaughan, 1979): (1) the C6 polypeptides are membrane proteins with molecular weights coincident with those of G/F, (2) the ADP-ribosylation of these peptides is cholera toxin dependent, (3) the cholera toxin catalyzed ADP-ribosylation is stimulated by GTP, and (4) the migration of the polypeptides in two-dimensional gels is similar to that of G/F from other sources (Schleifer et al., 1980; Kaslow et al., 1981a).

Under the incubation conditions that resulted in ADP-ribosylation of G/F, cholera toxin also catalyzed the ADP-ribosylation of several proteins including polypeptides that resembled tubulin in size, abundance, and subcellular distribution. Some of the cholera toxin catalyzed ADP-ribosylation products migrated in two-dimensional gels as acidic satellites

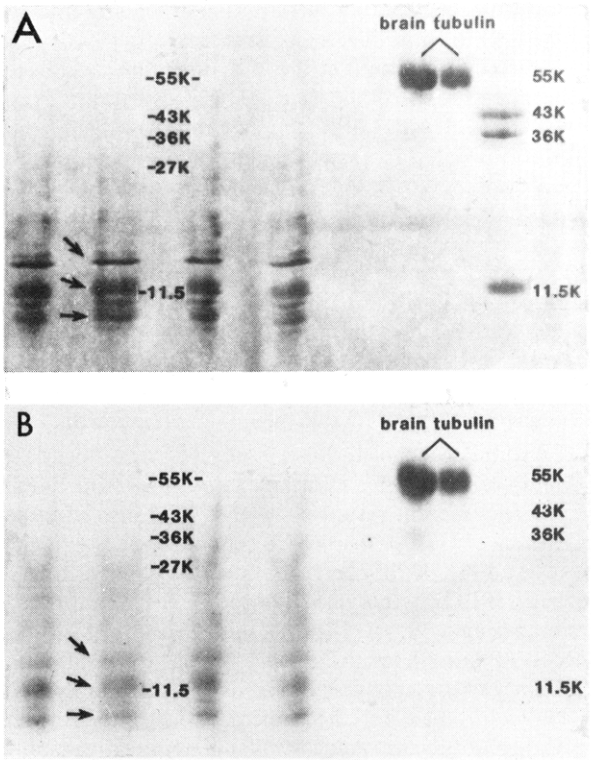

FIGURE 4: Peptide map of bovine brain tubulin and the associated [^{32}P]ADP-ribosylated protein. (A) Coomassie-stained gel. Incubation was with cholera toxin, GTP, and [^{32}P]NAD$^+$ (see Materials and Methods). The brain tubulin and the [^{32}P]ADP-ribosylated protein were subjected to limited digestion with *S. aureus* strain V8 protease. The four lanes on the left are replicate V8 protease treated samples. The position of undigested brain tubulin is indicated at the right. Molecular weight markers are ovalbumin at 43K, LDH at 36K, and cytochrome *c* at 11.5K. The positions of V8 protease and tubulin, known to be 27K and 55K, respectively, are also indicated. (B) Autoradiogram of the peptide map in (A). Arrows are present to indicate the correspondence between autoradiographic images produced by the [^{32}P]ADP-ribosylated peptide fragments and the Coomassie-stained tubulin peptide fragments.

to Coomassie-stained α and β tubulin of samples of either C6 cell cytosol or bovine brain tubulin (Figures 2 and 3). Finally, peptide mapping results demonstrated an identity between the [^{32}P]ADP-ribosylated and Coomassie-stained brain tubulin peptide fragments. This result strongly supported the conclusion that tubulin per se was ADP-ribosylated.

Kaslow et al. (1981b) have reported that cholera toxin catalyzed the transfer of ^{32}P from NAD$^+$ to human foreskin fibroblast proteins that had been extracted with buffered Triton X-100. The ^{32}P-labeled proteins were resolved in two-dimensional gels as satellites on the acidic side of a constellation of proteins believed to include intermediate filament protein (IFP) and tubulin. When purified, chick brain tubulin was similarly incubated and electrophoresed, several ^{32}P-labeled proteins were found in positions corresponding to those of labeled peptides from fibroblast extracts. The conclusion was drawn that tubulin and perhaps a microtubule-associated protein were ADP-ribosylated. It was also concluded that cytoskeletal IFP could be ADP-ribosylated in vitro. The results of the present report are consistent with and extend the observations of Kaslow et al. (1981b).

We are unable to demonstrate cholera toxin dependent ADP-ribosylation in intact C6 cells labeled for 24 h with either [^{32}P]P$_i$ or [2,8-^{3}H$_2$]adenine and analyzed by autoradiography following two-dimensional gel electrophoresis. However, we did observe the cholera toxin stimulated ^{32}P labeling of the intermediate filament protein vimentin, previously described

as a cAMP-dependent phosphorylation product in intact C6 cells (Groppi & Browning, 1980; Browning & Sanders, 1981). The observed charge shift of the [^{32}P]vimentin was indicative of phosphorylation rather than ADP-ribosylation. Nevertheless, a small fraction of tubulin might be modified in intact C6 cells, but only to an extent below the limits of detection using present methods (i.e., less than 0.1% of the total cellular tubulin could be ADP-ribosylated). Endogenous ADP-ribosyl transferase activity capable of transferring label from [^{32}P]-NAD^+ to C6 membrane G/F or cytosolic tubulin was not observed, although a toxin-independent M_r 38 000 labeled product was observed in vitro (Figure 1, lanes B and C). ADP-ribosyl transferase activity, which can mimic the effects of cholera toxin, has been reported in avian erythrocytes (Moss & Vaughan, 1978) and thyroid membranes (Vitti et al., 1982).

GTP stimulates cholera toxin catalyzed ADP-ribosylation of G/F in other systems (Watkins et al., 1981; Enomoto & Gill, 1980). Present results show that GTP also stimulates the labeling of G/F protein of C6 cell membranes (Figure 1, lanes G and H). GTP also stimulated the cholera toxin dependent [^{32}P]ADP-ribosylation of tubulin in mixtures of C6 membranes and cytosol (Figure 1, lanes I and J). Although added GTP stimulated ADP-ribosylation of C6 tubulin in membrane/cytosol mixtures, we have not conclusively shown a GTP *requirement* for the cholera toxin catalyzed ADP-ribosylation of tubulin. Added GTP did not greatly stimulate labeling of samples of cytosol alone (Figure 1, lanes D and E) in which the cytosol was 8-fold more concentrated than in samples of membrane/cytosol mixtures. However, demonstrating GTP effects in such experiments can be complicated for several reasons: (1) endogenous guanyl nucleotides are likely present in the subcellular fractions, (2) GTP may significantly contaminate ATP (Kimura et al., 1976) included in all incubations, and (3) guanyl nucleotides may remain tightly bound to tubulin (Spiegelman et al., 1977).

The G/F protein, eukaryotic initiation factor 2 (eIF-2), and a retinal "GTPase" can each be ADP-ribosylated by cholera toxin (Cooper et al., 1981). In addition, eukaryotic elongation factor 2 (EF-2) is ADP-ribosylated by diptheria toxin (Pappenheimer, 1977). These proteins possess GTPase activity and undergo regulatory GDP/GTP exchange. Although a nonidentity between the ADP-ribosylated and GTP-binding subunits may exist [i.e., eIF-2 (Cooper et al., 1981)], the proteins may share domains that can undergo cholera toxin catalyzed ADP-ribosylation, resulting in modified interaction of these proteins with modulatory guanyl nucleotides. Tubulin is also a GTP-binding protein that may hydrolyze GTP (Timasheff & Grisham, 1980). The M_r 110 000 heterodimer of α and β tubulin has two GTP-binding sites: one exchangeable and one nonexchangeable (Weisenberg et al., 1976; Spiegelman et al., 1977). Photoaffinity labeling studies demonstrate an exchangeable GTP-binding site on β but not α tubulin (Geahlen & Haley, 1977). It is not clear whether the nonexchangeable GTP-binding site is on α or β tubulin. Our results lend support to the idea that there is some common structural and/or functional feature shared by such GTP-binding proteins related to sites that are susceptible to ADP-ribosylation.

While much remains to be learned about microtubule stability and polymerization/depolymerization reactions, it is clear that GTP binding and perhaps GTP hydrolysis influence the stability of microtubules (Timasheff & Grisham, 1980). ADP-ribosylation of G/F results in altered GTPase (Cassel & Selinger, 1977) and/or GDP/GTP exchange (Lad et al., 1980) and a consequent change in adenylate cyclase activity. ADP-ribosylation of EF-2 and eIF-2 effects a change in GTPase activity and/or GDP/GTP exchange, resulting in a consequent inhibition of protein synthesis (Pappenheimer, 1977; Cooper et al., 1981; Jagus & Lin, 1981). It will be of interest to learn if the cholera toxin catalyzed ADP-ribosylation of tubulin can effect microtuble stability or polymerization/depolymerization reactions.

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Absorption Spectral Properties of Acetylated Bacteriorhodopsin in Purple Membrane Depending on pH†

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ABSTRACT: The dark-adapted form of bacteriorhodopsin in the purple membrane of *Halobacterium halobium* changes its absorption maximum from 560 to 600 nm if the pH is lowered to about 2 [Oesterhelt, D., & Stoeckenius, W. (1971) *Nature (London), New Biol. 233*, 149; Moore, T. A., Edgerton, M. E., Parr, G., Greenwood, C., & Perham, R. N. (1978) *Biochem. J. 171*, 469; Mowery, P. C., Lozier, R. H., Chae, Q., Tseng, T.-W., Taylor, M., & Stoeckenius, W. (1979) *Biochemistry 18*, 4100; Fischer, U., & Oesterhelt, D. (1979) *Biophys. J. 28*, 211; Muccio, D. D., & Cassim, J. Y. (1979) *J. Mol. Biol. 135*, 595]. We compared the pH dependence of the absorption spectra of acetylated membrane with that of unacetylated native membrane. The completely acetylated membrane showed a midpoint of pH 4.8 for the conversion to the acidic form; that of the native membrane was 3.4. On acetylation, the absorption maximum at neutral pH moved from 560 to 555 nm with about 20% decreases in extinction coefficients as compared with that of the native membrane, whereas the spectrum in acid was not affected. The chloride-dependent blue shift from the acidic form of the acetylated membrane was largely suppressed. The CD spectrum of the acetylated membrane was composed of two bands of an opposite sign with slightly decreased amplitudes. The chromophore of the acetylated membrane was sensitive to hydroxylamine, and the spectrum before bleaching was restored on addition of *all-trans*-retinal to the bleached membrane followed by dark incubation. Blue light irradiation accelerated the conversion to the acidic form in the native membrane but not in the acetylated membrane. Reductive ethylation did not affect the pH dependence of the absorption spectra.

Bacteriorhodopsin (bR)[1] in the purple membrane (PM) of *Halobacterium halobium* carries out a unidirectional shift of protons across the membrane with the aid of light energy absorbed by the retinylidene chromophore (Stoeckenius et al., 1979). These protons are supposed to be conveyed along a series of proton binding groups spanning the membrane as has been postulated for the proton fluxes through the channel of proton-dependent ATP synthetase (Sone et al., 1979). In this respect it would be interesting if chemical modifications of charged residues could affect the spectral properties of the chromophore. Lemke & Oesterhelt (1981) have shown the presence of tyrosine-26 in the vicinity of the chromophore.

Oesterhelt & Stoeckenius (1971) early found the bathochromic shift of the visible absorption spectrum when PM was placed in a weakly acidic medium. Since then, the acidic form of bR has been studied from various aspects: a close examination of its pH dependence (Moore et al., 1978; Mowery et al., 1979; Fischer & Oesterhelt, 1979), its relation to the "O" intermediate in the photocycle of bR (Moore et al., 1978; Fischer & Oesterhelt, 1979; Edgerton et al., 1980), and photoreactions and the analysis of the products (Mowery et al., 1979; Maeda et al., 1980, 1981; Fischer et al., 1981).

Acetylation with acetic anhydride is known to be one of the conventional methods for chemical modification of proteins (Riordan & Vallee, 1972) and is useful, especially in view of its narrow specificity restricted to lysine residues in the case of PM as discussed by Takeuchi et al. (1981). Moreover, acetylation induces pronounced decreases in both light-dependent activities of the proton release from the membrane sheets and of the proton transport across the membrane (Takeuchi et al., 1981). The present studies deal with the effect of acid on the acetylated PM along with its spectral characteristics. The effect of light on the formation of the acidic form of PM is described.

Materials and Methods

PM sheet fragments were prepared by a standard method described by Oesterhelt & Stoeckenius (1974). Acetylation was carried out as described by Takeuchi et al. (1981). PM suspensions (A_{560nm} = ~4) was mixed with an equal volume of saturated solution of sodium acetate. A 10-μL portion of acetic anhydride to every 5 mL of the reaction mixture was added at 10-min intervals with constant stirring at 0 °C. The pH was maintained between 7.5 and 8.0 by adding 1 M NaOH. Acetylation was terminated by diluting with ice-cold water at 80 min unless otherwise specified. The acetylated membrane was precipitated by centrifugation at 34000*g* for 30 min and washed twice with water. Pellet was suspended in water, and the suspensions were dialyzed against 5 mM

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[1] Abbreviations: bR, bacteriorhodopsin; PM, purple membrane; TNBS, 2,4,6-trinitrobenzenesulfonic acid; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; A-PM, acetylated purple membrane; U-PM, unacetylated purple membrane; CTAB, cetyltrimethylammonium bromide.

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